`

INVESTIGATION ON THE EFFECT OF ARTIFICIAL CHAPERONES ON LYSOZYME AGGREGATION

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by

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CERTIFICATE

This is to certify that the thesis entitled **"INVESTIGATION ON THE EFFECT OF ARTIFICIAL CHAPERONES ON LYSOZYME AGGREGATION** "by AVISHEK PARIHARY (110BM0633) submitted to National Institute of Technology, Rourkela for the degree of Bachelor in Technology is a record of bonafide research work, has been carried out by him in the Department of Biotechnology and Medical Engineering under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other university/institute for the award of any degree or diploma.

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ABSTRACT

Protein misfolding and aggregation is an unwanted phenomenon occurring in cells which is one of the cause of various degenerative diseases. The conformational changes occurring due to certain environmental conditions and due to the mutations in the native state causes large amount of β sheets which are stable in the body environment because of their lower energy state. Once amyloid formation takes place lysis of these molecules becomes really difficult. Hence it is very important to stop aggregation at its very early stage.

In this work modification was done on existing protocols on protein aggregation and an altogether new protocol was developed .Using this protocol, which involved denaturing of lysozyme protein at high temperatures, interference of molecules like oleic acid and sucrose were observed on the aggregates formed. Thioflavin T assay and Congo red assay were performed .Observations were monitored and recorded using fluorescence spectrophotometer and UV/VIS spectrophotometer.

Oleic acid was found to accelerate the aggregation mechanism whereas sucrose was found to inhibit it. This important analysis gave important information about molecules that can be administered as drugs for inhibiting protein aggregation.

Key words: *Protein mis-folding, protein aggregation, spectrophotometer.*

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CHAPTER 1

INTRODUCTION

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1.1. INTRODUCTION:

A polypeptide chain consisting of basic amino acids joined by a peptide bond forms the primary structure. Within the long protein chains there are regions in which the chains are organised into regular structures known as alpha-helices (alpha-helixes) and beta-pleated sheets. These are the secondary structures in proteins. These secondary structures are held together by hydrogen bonds. The tertiary structure of a protein is a description of the way the whole chain (including the secondary structures) folds itself into its final 3-dimensional shape. Proteins are folded and held together by several forms of molecular interactions. The molecular interactions include the thermodynamic stability of the complex, the hydrophobic interactions and the disulphide bonds formed in the proteins. The biggest factor in a proteins ability to fold is the thermodynamics of the structure. The interaction scheme includes the short-range propensity to form extended conformations, residue-dependent long-range contact potentials, and orientation-dependent hydrogen bonds.

The thermodynamics are a main stabilizing force within a protein because if it is not in the lowest energy conformation it will continue to move and adjust until it finds its most stable state. The next type of interaction in protein folding is the hydrophobic interactions within the protein (Acharya and Taniuchi, 1982). The thermodynamics play an important role in secondary structure formations while hydrophobic hydrophobic interactions affect the tertiary structure mainly (Arakawa and Tsumoto, 2003). These hydrophobic interactions also have an impact on the primary structure.

Globular proteins acquire distinct compact native conformations in water as a result of the hydrophobic effect. Hydrophobic cores are generally present in correctly folded state which are the basis of channel formation within the cell (Arakawa and Tsumoto, 2003). The hydrophobic interactions are shown to have an impact on the protein even after it has found the most stable conformation in how the proteins can interact with each other as well as folding themselves (Tsumoto et al., 2004).

Disaggregation of proteins is caused by a special class of molecules called the HSP (Heat Shock Proteins). Protein misfolding is a common property among proteins and is dependent upon various conditions. The main amino acid sequence and certain mutations accelerate the process. Moreover, it also depends on environmental conditions, because once proteins are exposed to specific environmental changes such as increased temperature, high or low pH, agitation, elevated glucose,

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or oxidative agents, they can lose their native conformation more rapidly. The loss of the native state of the proteins causes their denaturation and leads to unfolding. Because of the lack of arrangement, unfolded proteins are non-functional as they have lost the 3 dimensional tertiary structure which defines their function (Ventura and Villaverde, 2006).

Importantly, the unfolded state is thermodynamically unfavourable and unstable. Seeking lower energy levels and more stability, unfolded proteins have a tendency to aggregate and attain a state of lowest energy level and become thermodynamically stable. During aggregation, proteins change dynamically and most of the alpha helical structures change into cross-β structure, including intermediates varying from unordered amorphous aggregates to ordered fibrils that are called amyloid (Tsumoto et al., 2004). Subsequent to protein misfolding, aggregation, which consists of two parts, starts. Nucleation is the first part, when proteins reversibly attaches to a growing hydrophobic core. The second part is when other protein molecules attach themselves to this core. The formation of large number of β-sheet and high concentration makes the aggregate highly stable. Low net charge also is one of the important initiators of protein aggregation because it provides stability and lower energy state. Depending on the protein, there are various alternatives concerning how the precursor of aggregation is generated from native proteins. Incomplete protein degradation and introduction of any misfolding variant is the main reason of development of a precursor (Tsumoto et al., 2004).

1.2. Various models on protein aggregation:

1.2.1. Lumry – Eyring Two State Model:

This is a model for protein aggregation. According to this model, the native protein undergoes first a reversible conformational change to an aggregation prone state, which subsequently assembles irreversibly to the aggregated state (Ma et al., 2003).

1.2.2. Polymerization model:

In this model, misfolding occurs as a consequence of protein aggregation which follows a crystallization-like process dependent upon nucleus formation. The nucleus that is formed is the base of aggregation formation (Soto, 2001).

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1.2.3. Conformational model:

This model proposes that induced conformational changes results in the formation of the misfolded protein caused due to mutation of the native state that may or may not aggregate. Amyloid formation is not a mandatory step in this model (Soto, 2001).

1.2.4. Oligomerization model:

Intermediate formation takes place due to slight conformational change. This intermediate is unstable due to the presence of hydrophobic chores and high energy state. The formation of β sheets by interaction with other such intermediates stabilizes this state, which by further growth produces amyloids (Soto, 2001).

1.3. A summary of methods used for analysis of protein aggregates:

Table 1. Methods used for analysis of protein aggregates

Method	Application
SDS – PAGE SE-HLPC RP-HLPC	Size estimation and to distinguish from reducible covalent from non – covalent aggregates (Singh et al., 1991).
Capillary Electrophoresis Field Flow Fraction	Size estimation and quantification (Chien, 1991)
Static Light Scattering	Size and shape estimation (Brown, 1996)
Dynamic Light Scattering	Size distribution (Goldburg, 1999)
Analytical Ultracentrifugation	Size, shape estimation and quantification (Laue, 2001)
Coulter Counter	Size and member estimation (Bull et al., 1965)
CD Fluorescence Spectroscopy IR Spectroscopy NMR Resonance	Structural Analysis (Vliegenthart et al., 1983)

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1.4. OBJECTIVES:

- To modify existing protocols and to develop an altogether novel protocol for lysozyme aggregation.
- To investigate the effect of artificial chaperones on the aggregates formed.

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CHAPTER 2

LITERATURE REVIEW

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2.1. Hen egg white lysozyme :

Lysozyme is present in the mucosal secretion such as saliva and tears. In high concentration, about 3% from all proteins, Lysozyme is present in chicken egg-white. This enzyme is only effective against Gram positive bacterial cells. Gram negative bacteria and yeast are completely resistant to lysing by it. Molecular weight – 14307 Da and its isolectric point – 11.35 and optimum pH – 6-9 (maximum activity at 6.2) (Blake et al., 1967).

2.2.1. Substrates and inhibitors:

The natural substrate for lysozyme is the peptidoglycan layer of bacterial cell walls. However, to make various photometric, isotopic, and immunological lysozyme assays ,variety of low molecular mass substrates including murine degradation products as well as synthetic compounds have been used (Lee and Timasheff, 1981).

2.2.2. Compounds that affect protein aggregation:

Acridine derivatives, kaempferol, curcumin, surfactant coplymers like Polyethylene Oxide – Polypropylene Oxide – Polyethylene Oxide (PEO – PPO – PEO) inhibit aggregation while steel, glass, silicon rubber accelerate aggregation (Arakawa and Tsumoto, 2003).

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2.3. Artificial chaperones and their role in protein refolding:

Denaturation of tissue proteins following exposure to high temperatures or other various stresses is thought to be a major cause of tissue death. Thermo tolerance can be induced in biological cells (Kudou et al., 2003) . The molecular mechanism of this thermo tolerance involves increased biosynthesis of heat shock proteins that is the chaperone refolding of heat denatured proteins. The induction of heat shock proteins indicate that with the increase in concentration of chaperones the damage done to cells can be reversed. For burn and similar state injuries synthetic chaperones can be induced that play the role of natural chaperones and cause protein folding. In situations when normal cellular repair functions are incapable of managing the injury caused by heat, the availability of techniques to repair this damage is limited in the pharmaceutical industry. Therefore, for the treatment of such trauma and injuries there arises and inevitable need of creating synthetic molecular chaperones. It is well established that several human shock proteins can catalyse solubilisation and refolding of stable misfolded protein aggregates. The mechanism of refolding by human shock proteins is that they bind to the unfolded proteins to prevent or reduce

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The self-association of denatured proteins and prevent aggregation. In principle, unfolded proteins may spontaneously refold to their native states, or intermediate states, if their aggregation is prevented and this is targeted as the base trauma therapeutics (Krause et al., 2002).

2.4. Small molecule inhibitors of lysozyme aggregation:

Protein aggregation is associated with a number of degenerative human pathologies, but the precise mechanisms underlying the toxicity of amyloids and their cure is still under proper study. They are still incompletely understood. In this context, drugs capable in blockade of the aggregation and misfolding of amyloid genic proteins should be considered in strategies aimed at the development of novel therapeutic drugs (Kita et al., 1994). Human lysozyme variants have been shown to form massive amyloid deposits in the livers and kidneys of individuals affected by hereditary systems (systemic amyloidosis) (Arakawa and Tsumoto, 2003).Various studies clearly show that these small molecules can act as prototypes for the development of drugs against amyloidosis.

2.5. Inhibition of amyloid fibrillation of lysozyme by indole derivatives:

Amyloid deposits are associated with chronic neuronal and systemic pathologies, including Alzheimer's, Parkinson's and Huntington's diseases, transmissible spongiform encephalopathy, and type II diabetes. Whole proteins and protein fragments that form the amyloid structures have have very minute fibrilar shape, formed of a network of cross β-sheet structure, which is the result of a conformational change of the native protein structure. These proteins have a domain from small peptides (e.g. amyloid b-peptide, amylin and insulin), to natively unfolded proteins (e.g. a-synuclein) and natively folded monomeric proteins (e.g. lysozyme), or even protein assemblies (e.g. transthyretin). As there is no sequence similarity between these proteins that have large structural diversity, it has been suggested that amyloid formation may be a generic property of polypeptide chains. On the basis of this phenomenon efforts have been devoted to obtaining a better understanding of amyloids formation mechanisms and, in parallel, finding methods for intervention.

Common inhibitors may be present for various amyloids which is inferred from their common features. Accordingly, some reports suggest that there can be some inhibitory effects of small organic compounds on fibril formation by different proteins. Some of these inhibitors have been observed to affect conversion to oligomeric intermediates, which are considered to be the most harmful forms of these proteins for living cells. These small inhibitors can also disaggregate previously formed filaments of various proteins. Moreover, they may also stabilize the native form

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of aggregation oriented proteins by favouring the transformation kinetics towards native stable oligomeric states, and may possess antioxidant properties, thereby counter affecting the toxic effects of active oxidants which play a crucial role in aggregation. Usually, these molecules are effective at very low concentrations, and some, such as indole derivatives, are currently used in other related conditions, making them interesting leading structure candidates in amyloid-linked diseases.

There are still many uncertain points with regards to the detailed mechanisms of action of these compounds and the conditions under which they are functional against aggregation. Because protein fibrillation is entirely dependent on its sequence, and since hydrophobic and stackable residues participate mainly in the early stages of this process, it has been suggested that disruption of hydrophobic and ð-stacking interactions of aromatic residues may be involved which can lead to reduced aggregation. Indole derivatives can bind to proteins, but the nature of the derivative is very important in this regard.

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CHAPTER 3

MATERIALS AND METHODS

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3.1. Chemicals used:

Lysozyme (RM074-1G), Dibasic Sodium Phosphate: - NaH_2PO_4 (RM6382 -550G), Guanidine hydrogen chloride (MB-014-500G), Thioflavin T (RM-10365), Congo red (RM-500G) purchased from HIMEDIA pvt ltd. Monobasic Sodium Phosphate: - Na_2HPO_4 (61754905001730) purchased from MERCK pvt ltd.

3.2. Equipment used:

Fluorescence spectrometer (Perkin Elmer LS55) and UV/Visible spectrophotometer (Perkin Elmer λ-35) were used to monitor and record all the results obtained.

3.3. Method:

3.3.1. Preparation buffer stock:

A buffer stock was prepared which was used to maintain the pH. For preparation of 50 ml of .1 M sodium phosphate buffer 2.89 ml of 1 M sodium dihydrogen phosphate was taken and 2.12 ml of sodium monohydrogen phosphate was taken and then the volume was adjusted to 50 ml (Ho et al., 2003).

3.3.2. Preparation of stock Lysozyme solution:

1 mg/ml stock solution of lysozyme was prepared. For this 2 mg of lysozyme was taken and 1 ml of 100 μM of sodium phosphate buffer was added.

3.3.3. Tested Protocols used for Lysozyme aggregation:

3.3.2.1. Protocol 1:

The stock buffer solution was adjusted to 20 μM adjusting its pH to 6.3. To 10 μM solution of lysozyme 3 M guanidine hydrochloride solution was added. The solution was stirred using a magnetic stirrer at 60 rpm for 20 mins (Hevehan and De Bernardez Clark, 1997).

3.3.2.2. Protocol 2:

A solution containing 1mg/mL lysozyme and 50 mM sodium phosphate Buffer was prepared and the pH was adjusted to 7.16. 200 μL aliquot of the stock solution was taken in a microtube and was then placed in a water bath. Temperature was increased from 25°C to 90°C at 1°C /sec. It was stored at 25°C for 20 mins. It was then centrifuged at 15000 g for 20 mins at 25^{o}C.

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3.3.2.3. A complete novel protocol for aggregation:

A stock solution of 1mg/mL lysozyme was prepared in 50 mM sodium phosphate buffer and the pH was adjusted to 7.16. From lysozyme (1mg/ml) stock solution 200 µL was added to 1 mL of buffer It was then heated at 100°C for 20 mins in a water bath. It was incubated at 25°C for 20 more mins after heating (Goldberg et al., 1991).

3.3.4. Protocol used for studying the effect of oleic acid and sucrose on Lysozyme aggregation:

Two test tubes were taken and were marked "Before Boiling" and "After Boiling". 1mL of 1mg/mL lysozyme stock solution was taken in both test tubes. 100 µL of oleic acid having volume fraction= 1 was added to the test tube marked "Before Boiling". Both the test tubes were closed using cotton plugs and aluminium foil. They were kept in the water bath for 20 mins at 100°C. After heating, both the test tubes were stored for 20 more mins at room temperature to allow the oleic acid to act properly on the aggregates formed in the test tube marked "Before Boiling" and "After boiling" (De Bernardez Clark et al., 1999).

3.3.5. Thioflavin T assay:

In four test tubes markings were done: "C" (Control Solution), "A" (Aggregated Solution), "BB_1" (Addition of reagent before boiling), and "AB_1" (Addition of reagent after boiling). The test tubes were incubated in the dark. Test tube "C" 100 µL of contained native lysozyme, 2885 µL of sodium phosphate buffer and 15 µL of Thioflavin T dye. Test tube "A" contained 100µL aggregated solution, 2885µL of sodium phosphate buffer and 15 µL of Thioflavin T. Test tube AB_1 contained 100 µL of Sucrose or Oleic Acid, 100 µL of aggregated lysozyme solution, 2785 µL of sodium phosphate buffer and 15 µL of Thioflavin T dye. Test tube BB_1 contained 200 µL of solution containing Sucrose or Oleic Acid and the aggregated solution of Lysozyme, 2785 µl of sodium phosphate buffer and 15 µL of Thioflavin T dye (Brinkmann et al., 1992).

3.3.6. Congo Red assay:

In five test tubes the following markings were done: "CR" (Congo Red), "C" (Control Solution), "A" (Aggregated Solution), "BB_1" (Addition of reagent before boiling), and "AB_1" (Addition of reagent after boiling) .Incubation was done in the dark. In four test tubes markings were done: "C" (Control Solution), "A" (Aggregated Solution), "BB_1" (Addition of reagent before boiling), and "AB_1" (Addition of reagent after boiling). The test tubes were incubated in the dark.

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Test tube “C” 100 µL of contained native lysozyme, 2885 µL of sodium phosphate buffer and 15µL of CR dye. Test tube “A” contained 100µL aggregated solution, 2885 µL of sodium phosphate buffer and 15 µL of CR. Test tube AB_1 contained 100 µL of Sucrose or Oleic Acid, 100 µL of aggregated lysozyme solution, 2785 µL of sodium phosphate buffer and 15 µL of congo red dye. Test tube BB_1 contained 200 µL of solution containing Sucrose or Oleic Acid and the aggregated solution of Lysozyme, 2785 µL of sodium phosphate buffer and 15 µL of CR dye. Test tube “CR” contained pure congo red solution of 15 µL and 2985 µL of sodium phosphate buffer (Asano et al., 2002).

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CHAPTER 4

RESULTS AND DISCUSSION

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4.1. Thioflavin T assay on lysozyme aggregation by protocol 1:

The graph below shows that lysozyme aggregate solution at 0 h had the lowest peak. The discrepancy is that the peak of the control solution was found to be greater than that of the test solution at 0 h which completely contradicts the theory which says that the control solution should have the lowest peak value. This may be attributed to the presence of guanidine hydrochloride solution. Hence this protocol did not give the desired results.

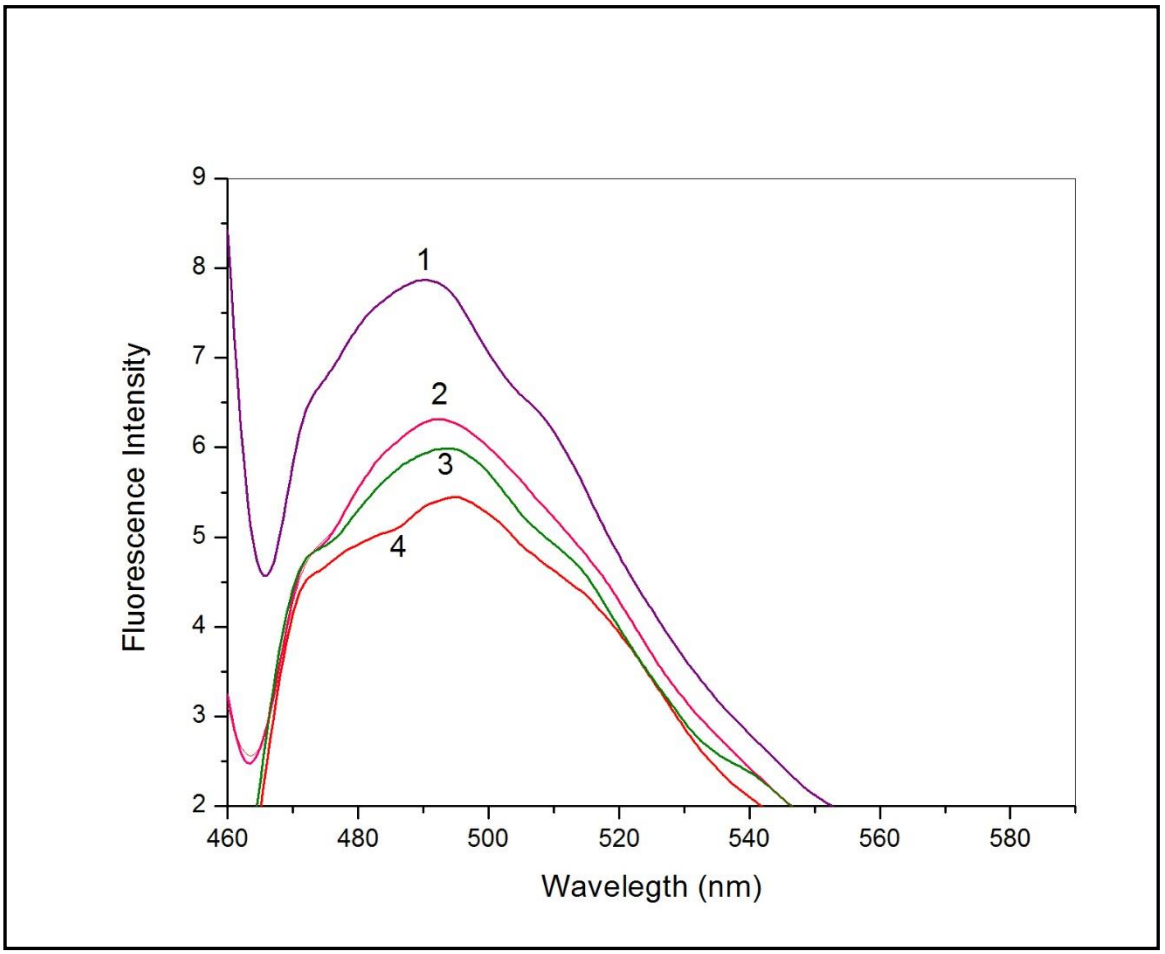

Figure 1. Graph depicting results of Thioflavin T assay performed on lysozyme aggregates formed by Protocol 1. (1) Lysozyme aggregate solution observed after 24 h. (2) Native lysozyme solution. (3) Lysozyme aggregate solution observed after 15 h. (4) Lysozyme aggregate solution observed after 0 h.

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4.2. Thioflavin T assay on lysozyme aggregation by protocol 2:

The graph below shows that the aggregation is less in the test solution (aggregated lysozyme solution) than in the control solution, which is absurd considering that control solution itself contains "non-aggregated" lysozyme. It can be inferred that the process of increasing the temperature from 25°C to 90°C gradually may not have supported the proper denaturation of lysozyme.

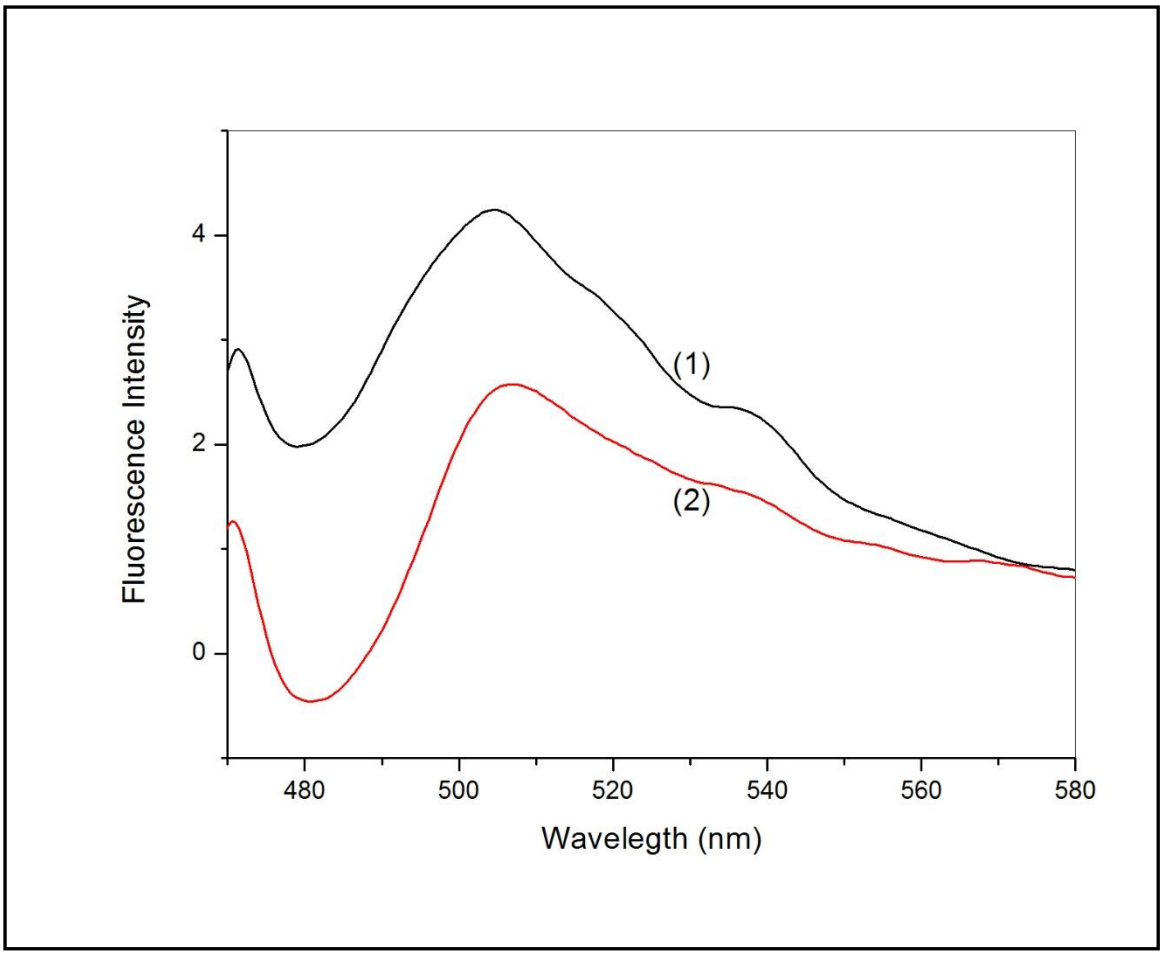

Figure 2. Graph depicting results of Thioflavin T assay performed on lysozyme aggregates formed by Protocol 2. (1) Native lysozyme Solution. (2) Aggregated lysozyme Solution.

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4.3. Congo red ssay on lysozyme aggregation by protocol 2:

No peaks were observed for control and test solutions in the Congo Red assay, although a peak was observed for the Congo Red solution. This indicated nothing about the formation of aggregates or amyloid formation. No peak formation in this case when compared to Thiflavin T assay results may be attributed to the less sensitivity of Congo Red.

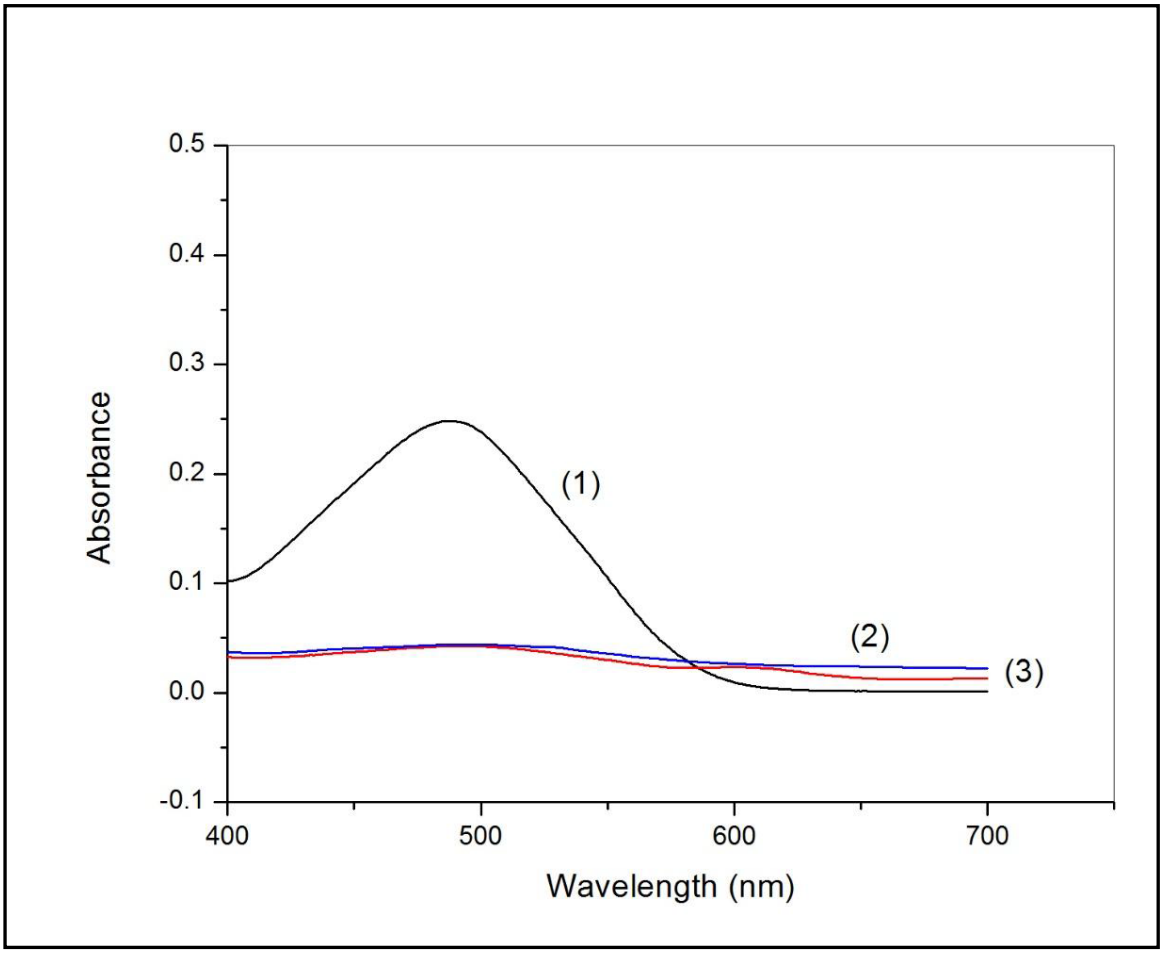

Figure 3. Graph depicting results of Cong Red assay performed on lysozyme aggregates formed by Protocol 2. (1) Pure Congo Red Solution.(2) Native lysozyme Solution.(3) Aggregated lysozyme Solution.

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4.4. Thioflavin T assay on lysozyme aggregation by protocol 3:

The graph below shows that the appearance of peak clearly shows the formation of aggregation. This may be caused due to the denaturing of the protein when heated to 100°C which causes the hydrophobic chores to be exposed and leads to hydrophobic-hydrophobic interactions within the molecule. Hence aggregation takes place**.**

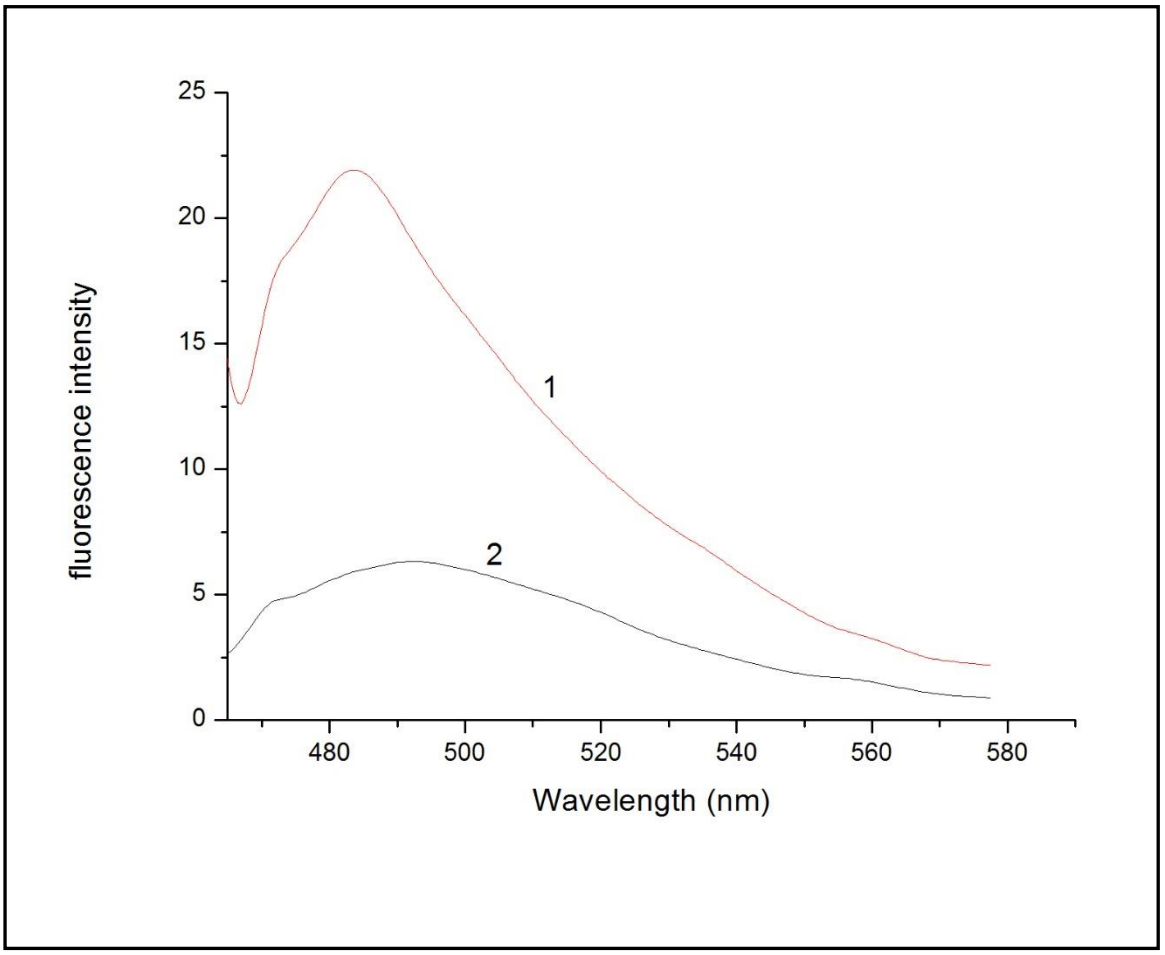

Figure 4. Graph depicting results of Thioflavin T assay performed on lysozyme aggregates formed by Protocol 3 (Novel protocol). (1) Aggregated lysozyme Solution. (2) Native lysozyme Solution.

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4.5. Congo Red Assay on lysozyme aggregation by protocol 3:

Results from Congo Red assay indicated that there is no amyloid formation as no shift in peak was observed. This protocol was dubbed as accurate and perfect for lysozyme aggregation under the laboratory conditions.

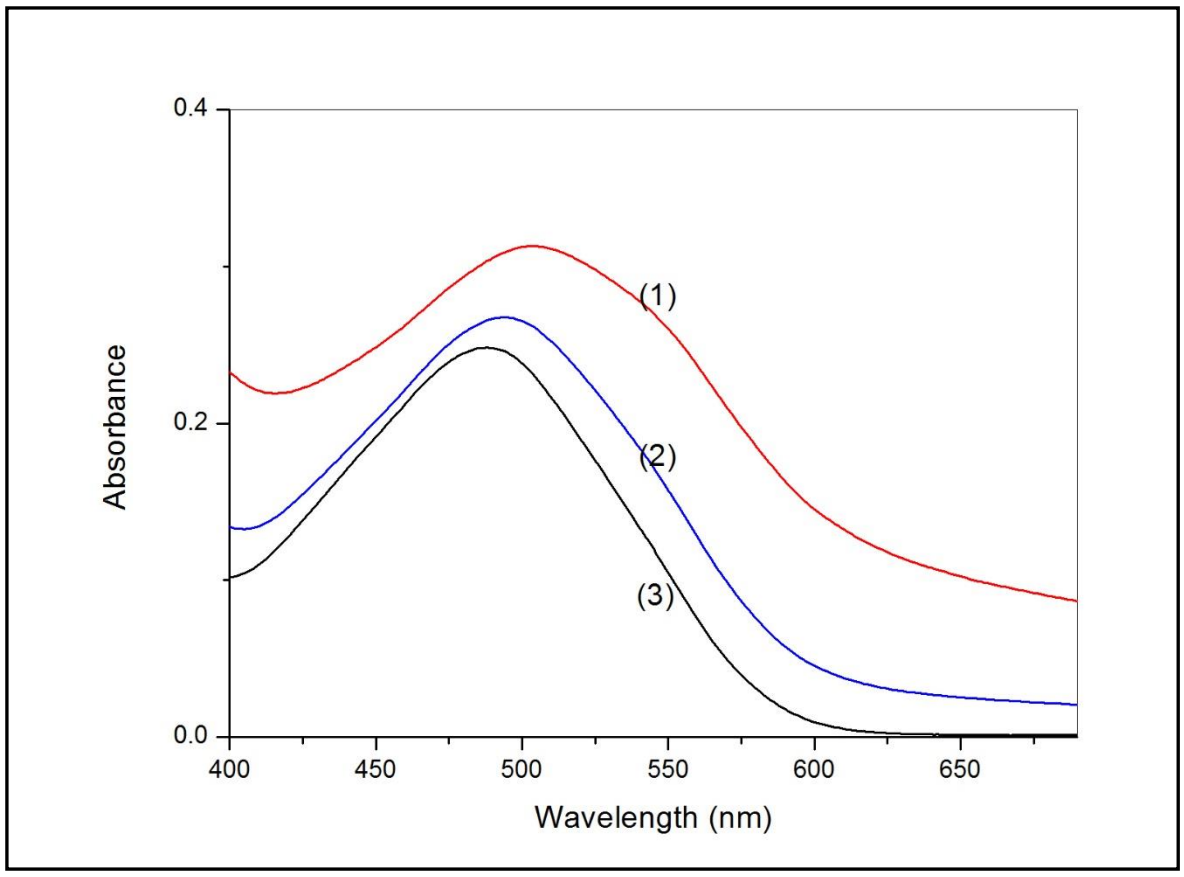

Figure 5. Graph depicting results of Congo red assay performed on lysozyme aggregate formed by Protocol 3. (1) Aggregated lysozyme Solution. (2) Native lysozyme Solution. (3) Pure Congo Red Solution.

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4.6 Thioflavin T Assay showing the effect of oleic acid on lysozyme aggregation:

As observed from the Thioflavin T assay, oleic acid significantly increased the aggregation in lysozyme, both when added before and after boiling. Oleic acid causes agitation by binding at sites favouring more hydrophobic hydrophobic interactions hence favours the denatured state more and causes acceleration in the process of lysozyme aggregation.

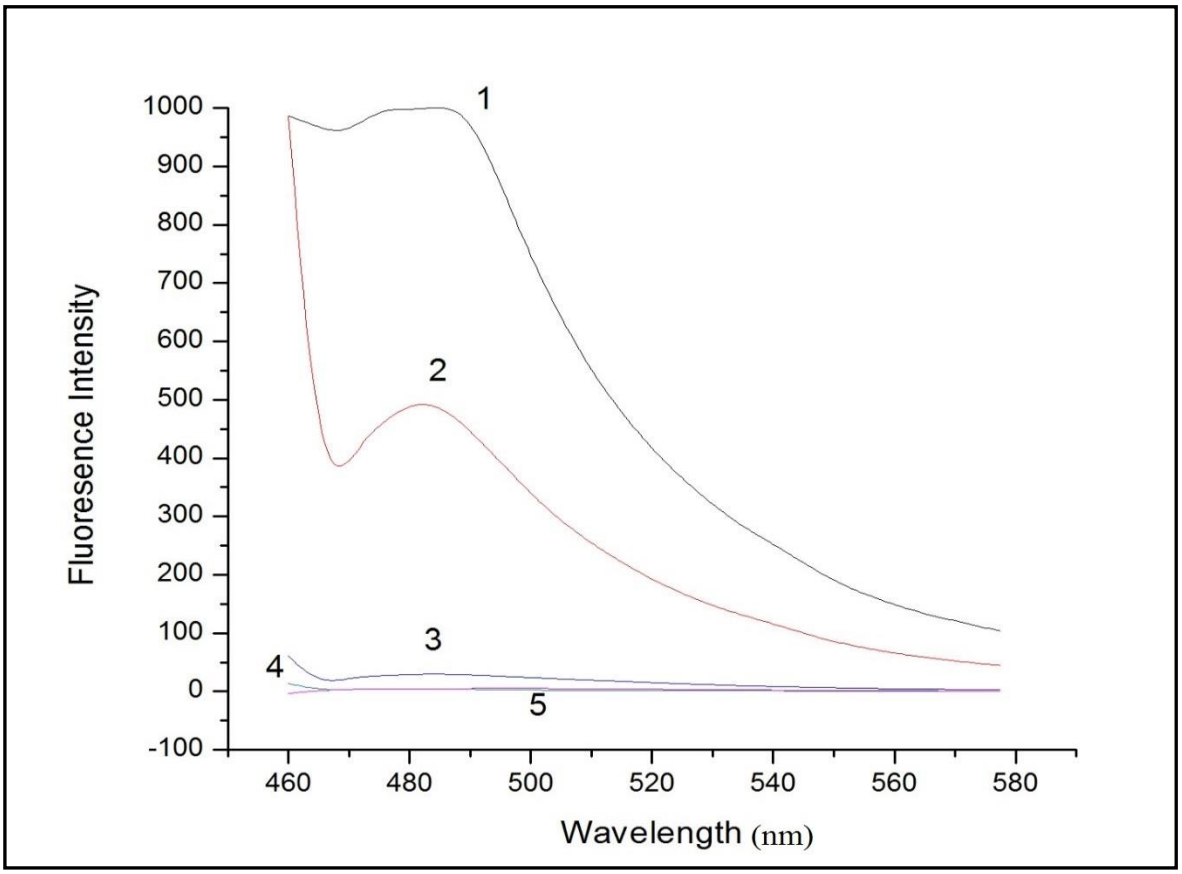

Figure 6. Graph depicting results of Thioflavin T assasy performed on aggregated lysozyme and oleic acid solution. (1) Aggregated lysozyme and oleic acid solution after boiling. (2) Aggregated lysozyme and oleic acid solution before boiling. (3) Aggregated lysozyme solution. (4) Native lysozyme solution. (5) Sodium Phosphate Buffer solution.

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4.7. Thioflavin T Assay showing the effect of sucrose on lysozyme aggregation:

The graph below shows that, sucrose significantly decreased the aggregation in lysozyme, both when added before and after boiling. This, again follows a simple mechanism. Poly solvents like sucrose increase the thermal stability of lysozyme and proteins in general.

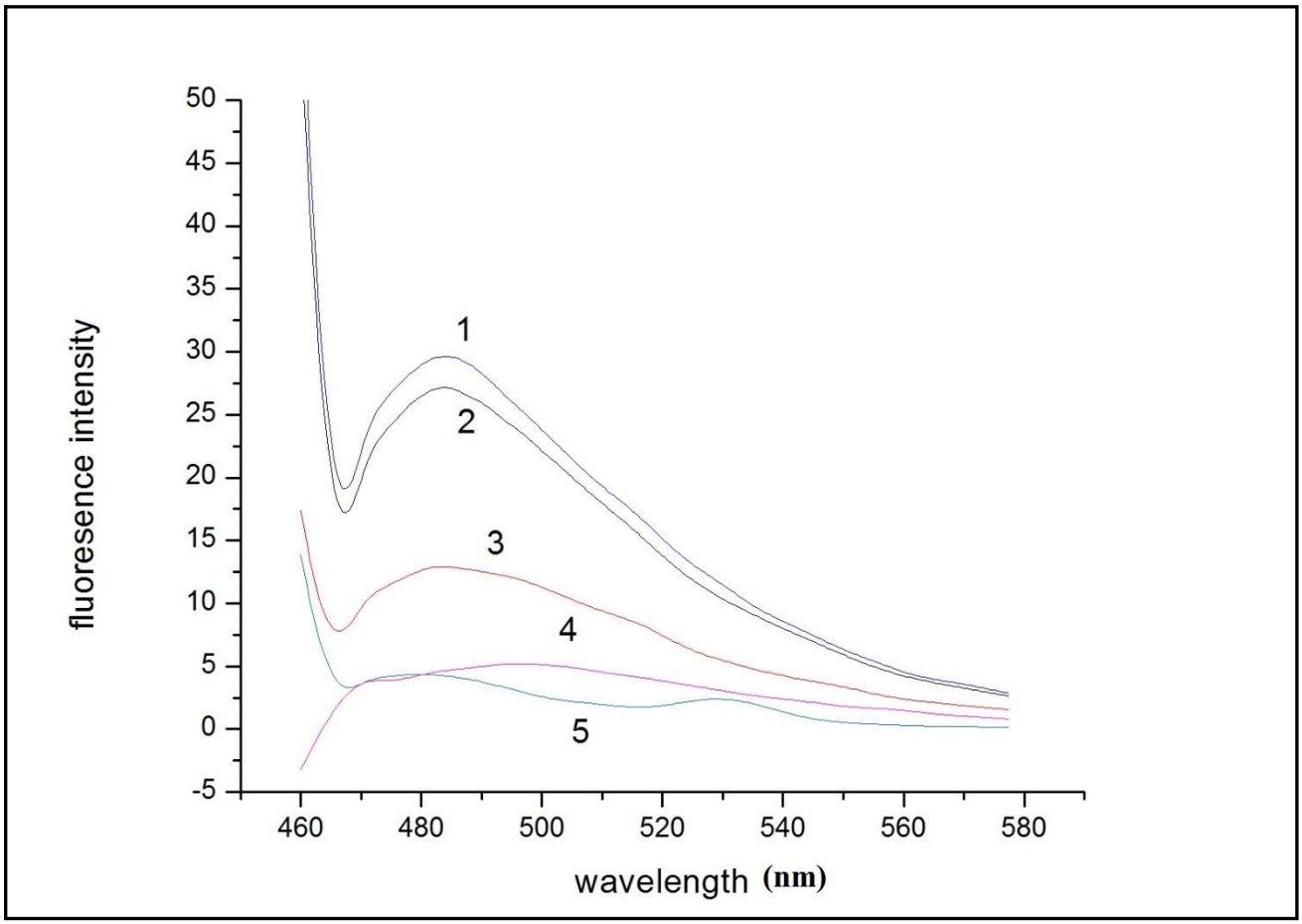

Figure 7. Graph depicting results of Thioflavin T assay performed on aggregated lysozyme and sucrose solution. (1) Aggregated lysozyme solution. (2) Aggregated lysozyme and sucrose solution before boiling. (3) Aggregated lysozyme and sucrose solution after boiling. (4) Native lysozyme solution. (5) Sodium Phosphate Buffer solution.

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4.8. Congo red Assay showing the effect of sucrose on lysozyme aggregation:

Again, no major peak shifts were observed in the Congo Red assay indicating that there was no amyloid formation in the solution.

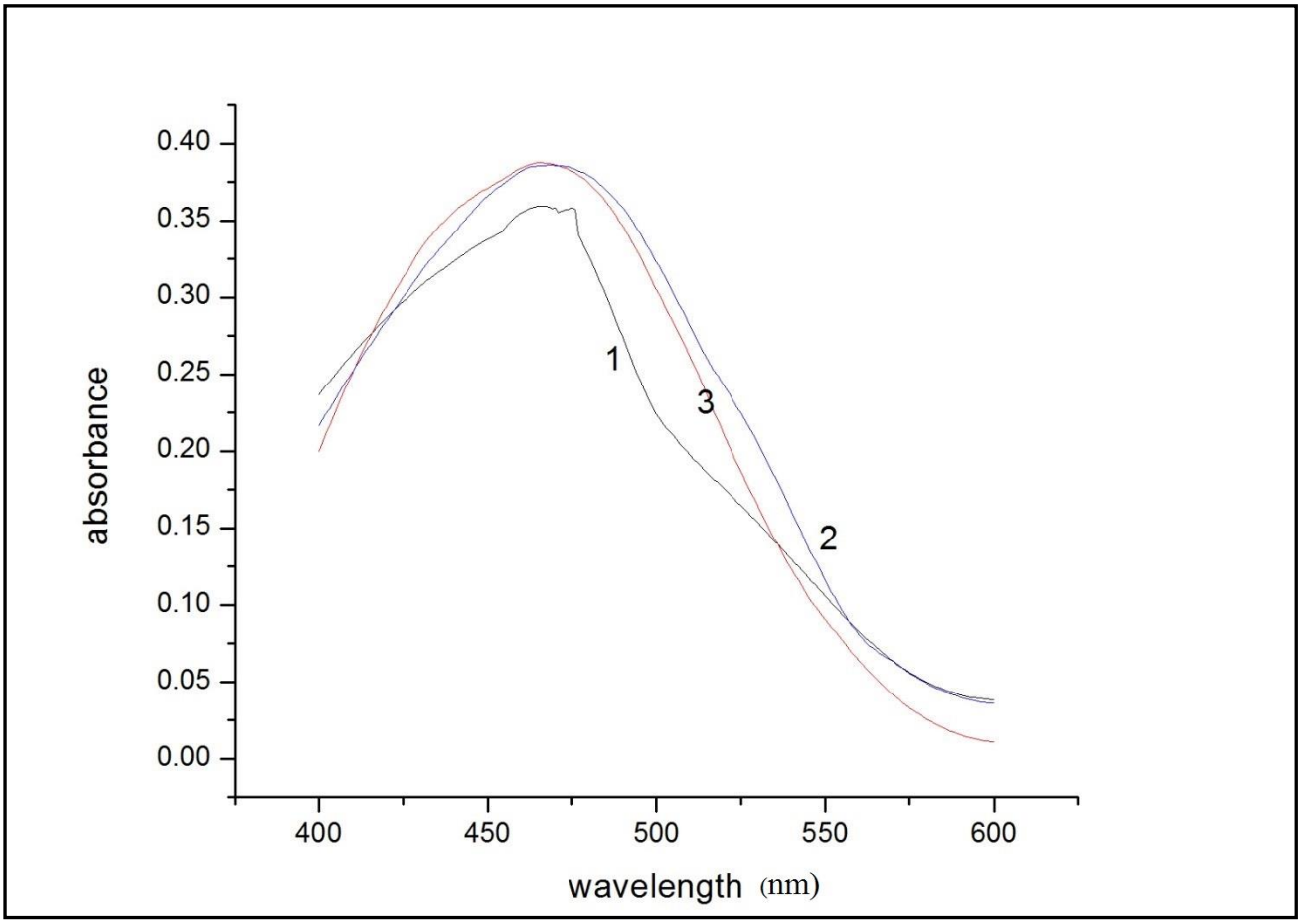

Figure 8. Graph depicting results of Congo Red assay performed on aggregated lysozyme and sucrose solution. (1) Aggregated lysozyme Solution (2) Aggregated lysozyme and sucrose solution before boiling (3) Aggregated lysozyme solution and sucrose before boiling.

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CHAPTER 5

CONCLUSION

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5.1. Conclusion:

With the use of congo red assay and Thioflavin T assay the aggregation of lysozyme was studied. At 100°C the aggregation was found to be immense. Sucrose was observed to be an inhibitor of aggregation of lysozyme. The reason of reduced aggregation can be related to the fact that sucrose caused less hydrophobic - hydrophobic interaction by binding to the core and thus favouring the native state over its denatured state. The energy of the native state is decreased and stability is increased. Oleic acid accelerated the process of aggregation both before and after boiling. Oleic acid being hydrophobic itself supports hydrophobic interactions within the protein molecule when its starts to denature due to heating. Lysozyme assembles into multimeric complexes in a solution with oleic acid which can also be considered as a reason for the increased aggregation observed.

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